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CONSTRUCTION OF SYSTEMS FOR DETECTION AND QUANTITATION BY UV-ABSORBING MOBILE-PHASE IONS IN REVERSED-PHASE CHROMATOGRAPHY

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SUMMARY

Ions without inherent detectable properties can be detected and quantified in reversed-phase liquid–solid systems by use of a mobile phase containing one or two UV-absorbing ions. The highest sensitivity is obtained when the sample and the detectable ion have approximately equal retentions. The system can be adapted to a given sample by the choice of solid phase and mobile-phase ions. A series of systems covering samples with widely different hydrophobicities is given. Retention can be regulated with an uncharged organic modifier, but this will decrease the detection sensitivity. Background absorbance above 0.5 will also decrease the sensitivity. The quantitation of a solute is not affected by the composition of the sample, *i.e.*, the solvent or other sample components.

INTRODUCTION

Ion-pair extraction in liquid–liquid systems has been used for many years for the quantitative spectrophotometric determination of compounds without absorbance or fluorescence of their own[1], and the technique has also been applied in liquid–liquid chromatography with organic mobile phases[2–8]. The principle is very simple: an UV-absorbing ion, applied in an aqueous stationary phase, gives ion pairs with samples of the opposite charge, which migrate with the organic phase.

The ion-pair distribution technique can also be applied for improvement of detection in reversed-phase systems with a hydrophobic adsorbent as the stationary phase[9,10]. An UV-absorbing or fluorescent ion is included in the mobile phase and distributed to the solid phase. Injected solutes will influence the distribution of the detectable ion by binding or displacement and give rise to a detector response, even when the solute lacks detectable properties. The solute peak can be positive or negative, depending on the retention and charge of the compound relative to the UV-absorbing mobile-phase ion (the probe). The latter also gives rise to peaks with constant retention (the system peaks), which can be positive or negative, depending on the nature of the injected sample.

This principle has been applied by Parris[11,12] for the detection of, *e.g.*, sur-

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factants with a quaternary ammonium ion or a sulphonate with aromatic substituents as the detectable mobile-phase component. Gnanasambandan and Freiser[13] have detected alkanols with methylene blue as the light-absorbing component in the system. Improvement of detection by UV-absorbing components in reversed-phase systems has also been reported by Bidlingmeyer[14], and Sachok *et al.*[15] have quantitated alkylsulphonates by the same technique.

Small and Miller[16] have used a related principle for the detection of inorganic ions with an ion exchanger as the stationary phase. The technique was called indirect photometric chromatography. The monitoring of inorganic anions has also been studied by Dreux *et al.*[17]. Stranahan and Deming[18] have used a computer simulation of so-called "induced peaks" in reversed-phase liquid chromatography. Bidlingmeyer and Warren[19] have studied the effects of ionic strength in the same kind of system.

Observations of a similar kind have also been made in systems without ion-pairing components in the mobile phase. Slais and Krejci[20] have demonstrated that a sample peak and a system peak with opposite directions are obtained when an organic compound is injected in a system with charcoal as adsorbent and cyclohexane with a low content of diethyl ether as the mobile phase. McCormick and Karger[21] have found a response of a similar kind in reversed-phase systems in which water with a low content of organic modifier was the mobile phase. Riedo and Kováts[22] have recently presented an extensive discussion of the theory for the formation of peaks in such systems.

Our previous studies[9,10] have shown that the detection sensitivity is highly dependent on the retention ratio between the solute and the detectable mobile-phase component. It has also been shown that a change in the concentrations of all mobile-phase ions occurs in the solute zone. The response can be considerably improved by using a mobile phase containing two detectable ions with different charges and hydrophobicities.

Studies on the relationship between the composition of the chromatographic system and the response to charged compounds are described in this paper. Methods for improving the detection sensitivity by adapting the system to the sample are discussed. A series of systems are presented that permit detection with good sensitivity of compounds with widely different hydrophobicities.

EXPERIMENTAL

Apparatus

The detectors used were an LDC Spectromonitor III and an LDC UV-III monitor. The latter was modified by placing an adjustable screw in the light path of the reference cell to enable compensation for high background absorbance in the sample cell. The pumps were a Gynkotek 600/200 and an Altex 100 A. Rheodyne 70-10 and 71-25 injectors were used. The columns (100 × 3.2 mm I.D. or 100 × 4.6 mm I.D.) were made of stainless steel with a polished inner surface, equipped with modified Swagelok connectors and Altex 250-21 filters.

The pH measurements were made with an Orion Research Model 801 instrument with Ingold Type 401 combined electrode. The spectrophotometric measurements were made with a Zeiss PMQ II Spektralphotometer.

Chemicals and reagents

Sodium salts of naphthalene-2-sulphonate (Eastman-Kodak, U.S.A.) and 6-hydroxynaphthalene-2-sulphonate (E. Merck, F.R.G.) were recrystallized from water before use. 1-Phenethyl-2-picolinium bromide was obtained from Eastman-Kodak and N,N-dimethylprotriptyline bromide was synthesized by Hässle (Sweden) from protriptyline (supplied by Merck, Sharp and Dohme, Stockholm, Sweden).

The salts were converted into the hydroxide or acid form by ion exchangers before use in the mobile phases. All other chemicals were of analytical-reagent grade.

Chromatographic system

μBondapak Phenyl (10 μm) (Water Assoc., U.S.A.), Nucleosil CN (10 μm) (Macherey, Nagel E Co., F.R.G) and PRP-1 (10 μm) (Hamilton Bonaduz, Switzerland) were used as solid phases. PRP-1 is a styrene–divinylbenzene copolymer, while the other phases are silica-based.

The mobile phases were aqueous solutions of ions giving a response in the detector, with additions of buffering compounds or other salts. In some instances methanol was added to the mobile phase.

Column preparation

The μBondapak Phenyl columns were packed by a slurry technique with water–ethanol (58:42) as suspending medium. They were washed with 200 ml of water–methanol (1:4) and, when tested with water–methanol (2:3) as mobile phase and toluene and mesitylene as retained samples, they gave capacity factors (k') of about 1 and 4, respectively.

The Nucleosil CN column was packed with chloroform as suspending medium, and washed with 400 ml of *n*-hexane. When tested with *n*-hexane–butanol (199:1) as mobile phase and 1,4-dinitrobenzene as retained compound, it gave $k' = 2$.

The PRP-1 column was packed with methanol as suspending medium, and washed with 200 ml of methanol. When tested with acetonitrile as mobile phase and toluene and naphthalene as samples, it gave $k' = 1$ and 2, respectively.

The columns gave reduced plate heights of 4–10 in the test systems.

Chromatographic technique

The eluent reservoir, injector, column and connecting tubes were thermostated at 25.0 ± 0.1°C in a water-bath.

The flow-rate was 0.5 ml/min. The mobile phase was not recirculated.

The samples were injected as solutions in the mobile phase unless stated otherwise. Peak areas were determined by planimetry.

The volume of mobile phase in the column, V_m, was obtained from the front peak of the chromatogram.

RESULTS AND DISCUSSION

Chromatographic response

The studies were performed with a number of different hydrophobic solid phases in combination with an aqueous mobile phase containing one or two detectable ions (probes), buffering components, aprotic ions and sometimes methanol as non-ionic modifier.

TABLE I

RESPONSE WITH ONE HYDROPHOBIC PROBE, S, IN THE MOBILE PHASE

Hydrophilic mobile-phase ions in large excess. Solute: A.

Charge of solute compared with S	*Solute peak*	
	$k'_A < k'_S$	$k'_A > k'_S$
Same or uncharged	Positive	Negative
Opposite	Negative	Positive

The ionic mobile-phase components, including the probe, are distributed to the solid phase with ions of opposite charge. A solute with suitable hydrophobicity will affect these distributions, and the detector shows the change in the probe concentration in the zone where the solute is eluted (solute peak) and in the zones that are characteristic for the mobile-phase (system peaks). The direction of the solute peak can be positive or negative, depending on the retention and charge of the solute relative to the probe, as shown in Table I[9,10]. This response pattern has been obtained when the probe is considerably more hydrophobic than the other (usually two) mobile-phase ions.

The concentration relationships between the mobile-phase ions are important. The normal response pattern (Table I) is obtained when the hydrophilic mobile-phase ions are present in significant excess. Deviations have been seen when the mobile phase contains only two ions. Table II gives results obtained with a solution of naphthalene-2-sulphonic acid in water as mobile phase. Anions give negative peaks, both before and after the system peak. The amines, which are more hydrophobic than the cation present in the mobile phase, are eluted after the system peak, but the carbon selectivity, which increases with increasing retention, is considerably lower than the separation factor of 3 per methylene group normally obtained[9].

A further case of deviating response is demonstrated in Figs. 1 and 2, obtained with a mobile phase containing one hydrophobic (1-phenethyl-2-picolinium, PEP) and four hydrophilic ions, none of them present in large excess. The measurements were made with two detectors coupled in series, one showing the change for 6-hy-

TABLE II

RESPONSE WITH TWO IONS IN THE MOBILE PHASE

Mobile phase: $3.7 \cdot 10^{-4}$ *M* naphthalene-2-sulphonic acid in water. Solid phase: μBondapak Phenyl. Amines applied as chlorides. Sulphonates and sulphates applied as sodium salts.

Substance	*Log k'*	Peak direction
Butyl sulphate	− 0.4	Negative
Hexanesulphonate	0.18	Negative
Naphthalene-2-sulphonate (system peak)	0.50	—
Octanesulphonate	1.11	Negative
Octyl sulphate	1.43	Negative
Propylamine	0.54	Positive
Butylamine	0.73	Positive
Pentylamine	0.99	Positive
Hexylamine	1.53	Positive

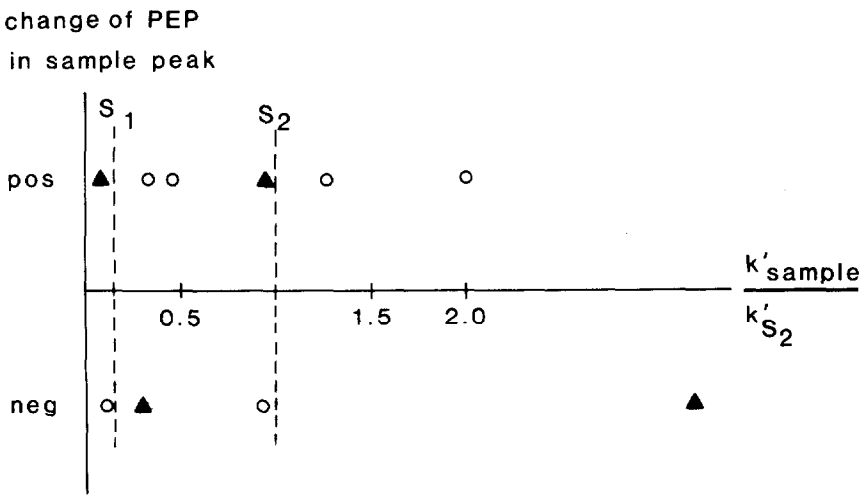

Fig. 1. Changes of 1-phenethyl-2-picolinium (PEP) in sample peak. Solid phase: μBondapak Phenyl. Mobile phase: 10^{-4} *M* 6-hydroxynaphthalene-2-sulphonate, $5.6 \cdot 10^{-5}$ *M* 1-phenetyl-2-picolinium, 10^{-3} *M* acetic acid, $4 \cdot 10^{-5}$ *M* Na^+; pH = 3.9. S_1 and S_2 = system peaks. ○, Anionic sample; ▲, cationic sample.

droxynaphthalene-2-sulphonate (6-OH-NS) at 300 nm ($\varepsilon_{300} = 2.5 \cdot 10^3$) and the other the changes at 261.5 nm for both 6-OH-NS ($\varepsilon_{261.5} = 3.4 \cdot 10^3$) and PEP ($\varepsilon_{261.5} = 4.8 \cdot 10^3$). From the chromatograms and the ε values for the two UV-absorbing ions (measured in the detectors used), the concentration changes for both UV-absorbing mobile-phase ions in the sample zones were evaluated. The retentions for the system peaks S_1 and S_2 are $k' = 2$ and 12.5, respectively.

A concentration change of the hydrophobic probe in the sample peak (PEP, Fig. 1) from positive to negative or *vice versa* occurs not only for retentions around

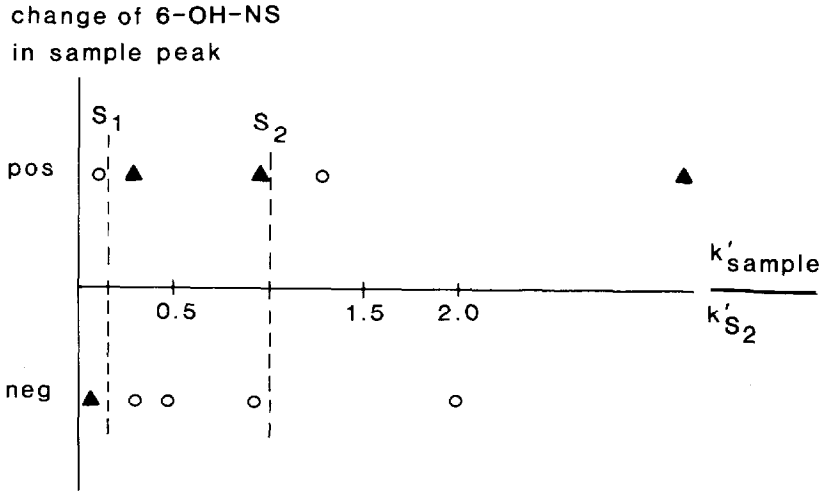

Fig. 2. Changes of 6-hydroxynaphthalene-2-sulphonate (6-OH-NS) in sample peak. Solid phase: μBondapak Phenyl. Mobile phase: as in Fig. 1. ○, Anionic sample; ▲, cationic sample.

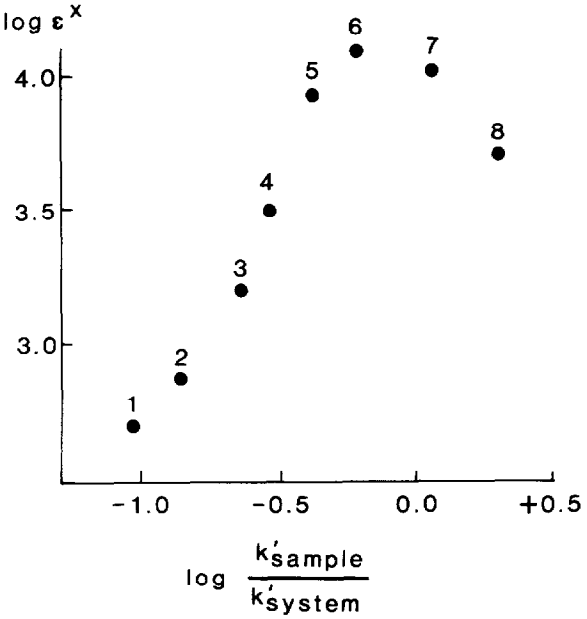

Fig. 3. Hydrophobic cationic probe. Solid phase: Nucleosil CN. Mobile phase: $1.8 \cdot 10^{-5}$ *M* N,N-dimethylprotriptyline in 0.01 *M* acetic acid. Detection: 292 nm. Sample: 1 = octylamine; 2 = octanesulphonate; 3 = octyl sulphate; 4 = decylamine; 5 = tetrabutylammonium; 6 = undecylamine; 7 = dodecylamine; 8 = tetrapentylammonium.

S_2, in agreement with Table I, but also at lower retention, between S_2 and S_1. The hydrophilic probe (6-OH-NS, Fig. 2) does not show the same change between S_1 and S_2. In sample peaks more retained than S_2, the changes of 6-OH-NS are small.

Fitting of the chromatographic system to a given solute

The probe ion must fulfill certain requirements to make it suitable for detection with high sensitivity: (1) it should have high detectability in the detector, *i.e.*, high molar absorptivity or fluorescence; and (2) it should have a retention similar to the solute with k' preferably in the range 5 10.

The relationship between the relative retention ($k'_{\text{sample}} / k'_{\text{system}}$) and the detection sensitivity obtained with a UV-absorbing cationic probe, N,N-dimethylprotriptyline, is shown in Fig. 3. The detection sensitivity is expressed by the conditional molar absorptivity, ε^x, which is the quotient between the found peak area, expressed in absorbance units, and the amount of compound injected:

$$\varepsilon^x = \frac{Y\ s\ u}{m\ d\ b}$$

where Y is the peak area, s is the sensitivity setting of the detector, u is the flow-rate, m is the number of moles of the compound, d is the chart speed and b is the path length in the detector cell.

Fig. 3 shows that the detection sensitivity has a maximum when the relative

retention is close to unity. However, a high response is obtained in a wide retention range, and a series of hydrophobic non-UV-absorbing compounds with relative retentions between 0.3 and 2 can be detected with a sensitivity that corresponds to a molar absorptivity between 3000 and 12000.

The solid phase must, as a rule, be adapted to the hydrophobicity of the probe, as other means of obtaining suitable retention of the probe can be used to only a limited extent. A selection of four systems, suitable for separation and detection of compounds within a wide hydrophobicity range, is given in Table III. μBondapak Phenyl is used as an adsorbent for the detection of moderately hydrophobic ions with probes such as naphthalene-2-sulphonate and 1-phenetyl-2-picolinium. The more hydrophilic Nucleosil CN is suitable for highly hydrophobic ions with the hydrophobic N,N-dimethylprotriptyline as probe. PRP-1 is a very hydrophobic adsorbent, which can be used in combination with the hydrophilic 6-hydroxynaphthalene-2-sulphonate as probe to detect samples with low carbon contents.

A solute can be detected by both cationic and anionic probes, but it can be advantageous to use a probe with a charge opposite to that of the sample, as it is then possible to adapt the retention of solute and probe to each other by changing the concentration of non-detectable ions in the mobile phase. An example is given in Fig. 4. An increase in the tetramethylammonium concentration increases the retention of the anionic probe naphtalene-2-sulphonate and decreases the retention of the cationic solutes (amines). Tetramethylammonium (0,02 *M*) gives about the same retention of solutes and probe, and this optimizes the detection sensitivity.

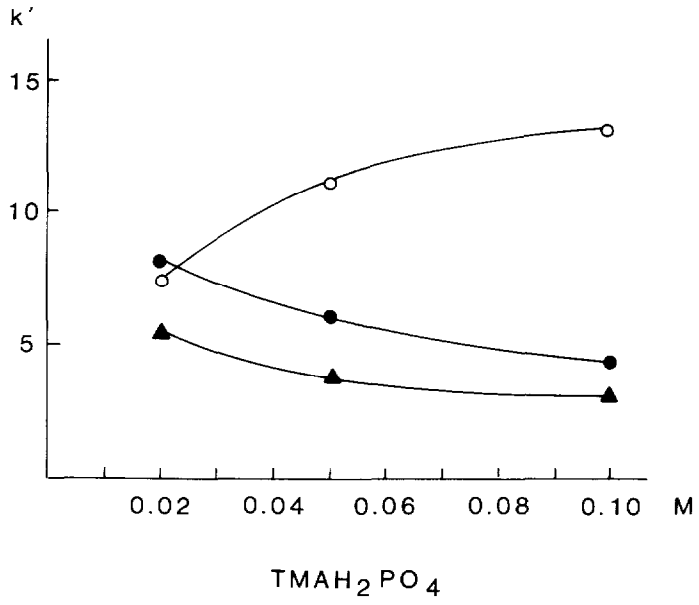

Fig. 4. Regulation of retention with counter ion. Solid phase: μBondapak Phenyl. Mobile phase: $3.7 \cdot 10^{-4}$ *M* naphthalene-2-sulphonate in tetramethylammonium phosphate buffer ($TMAH_2PO_4$); pH = 2.1. Detection: 254 nm. ○, Naphthalene-2-sulphonate; ●, dibutylamine; ▲, hexylamine.

TABLE III

DETECTION SYSTEMS FOR IONS OF DIFFERENT HYDROPHOBICITY

Probe	*Concentration in mobile phase (mol/l)*	*Solid phase*	*Solvent**	*Carbon content in sample*	
				Alkylamine	*Alkylsulphonate*
6-Hydroxynaphthalene-2-sulphonate	$5 \cdot 10^{-5}$	PRP-1	0.001 *M* NaOAc	2 %N 3	5
Naphthalene-2-sulphonate	$4 \cdot 10^{-4}$	μBondapak Phenyl	0.01 *M* H_3PO_4	5 7	6 8
1-Phenethyl-2-picolinium	$2 \cdot 10^{-4}$	μBondapak Phenyl	0.02 *M* TMAOAc	7 8	5 6
N,N-Dimethylprotriptyline	$2 \cdot 10^{-5}$	Nucleosil CN	0.01 *M* HOAc	9 12	9 – 12

* TMA = tetramethylammonium; OAc = acetate.

Influence of mobile-phase components on the sensitivity

The non-detectable ions in the mobile phase can have a strong influence on the response. The hydrophobicity and concentration of the ions with the same charge as the probe (co-ions) are of great importance, as illustrated in Fig. 5. The hydrophobic naphthalene-2-sulphonate is used as a probe, and the highest detection sensitivity is obtained when a hydrophilic co-ion, such as dihydrogenphosphate is present in low concentration. A more hydrophobic co-ion, such as pentanesulphonate, has a highly negative influence on the sensitivity, probably because it decreases the fraction of probe in the total amount of anions on the solid phase. Fig. 5 also shows that an increased binding of the probe, caused by adding a more hydrophobic counter ion, tetraethylammonium, decreases the detection sensitivity. Further examples of insensitivity due to high binding to the solid phase have been given in a previous paper[10].

Addition of a non-ionic modifier, such as methanol, is a useful way of decreasing the retention of both system and sample peaks without changing the relative elution order. Fig. 6 shows the response in three systems with the same concentrations of ions and 0, 5 and 10% of methanol, respectively in the mobile phase. The retentions of all peaks decreased about 3-fold when the methanol content was increased from 0 to 10%, but there was also a significant decrease in the detection sensitivity, especially before the system peak. Similar observations have been made by others[13]. The decrease might be due to the smaller amount of probe that is adsorbed on the solid phase when methanol is present in the system.

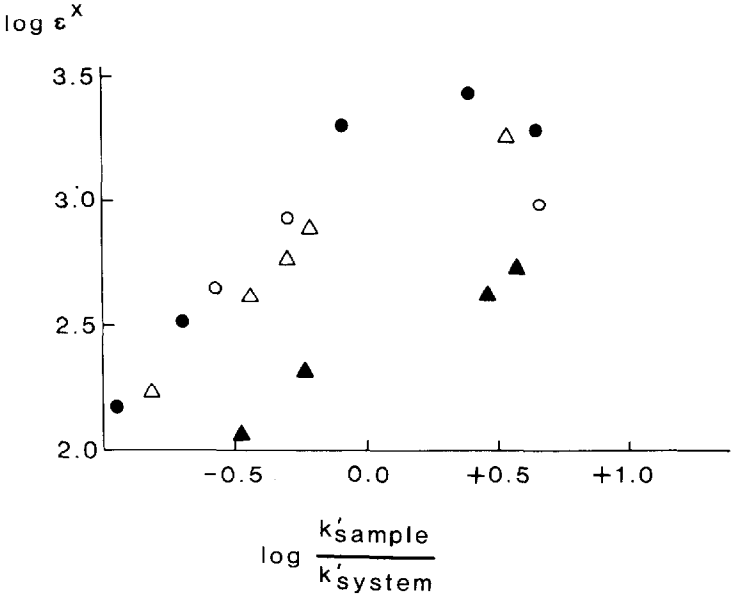

Fig. 5. Hydrophobic anionic probe: influence of non-detectable ions in the mobile phase. Solid phase: μBondapak Phenyl. Mobile phase: $4 \cdot 10^{-4}$ *M* naphthalene-2-sulphonate in ●, 0.01 *M* phosphoric acid; ○, 0.05 *M* phosphoric acid; ▲, 0.012 *M* sodium pentanesulphonate; △, 0.017 *M* tetraethylammonium acetate, pH = 5.4. Detection 254 nm. Sample: alkylamines and alkylsulphonates.

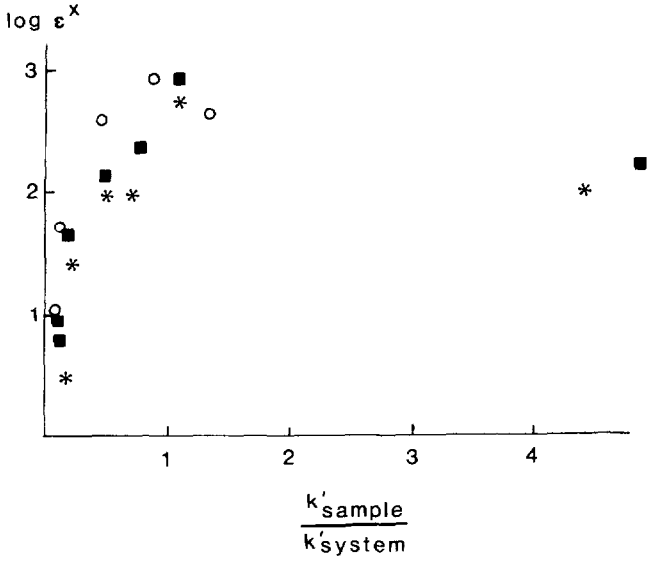

Fig. 6. Regulation with non-ionic modifier. Solid phase: PRP-1. Mobile phase: $5.1 \cdot 10^{-5}$ *M* 6-hydroxy-naphthalene-2-sulphonate, 0.01 *M* acetic acid, sodium hydroxide to pH = 6. Methanol content: ○, 0; ■, 5; ⁎, 10%.

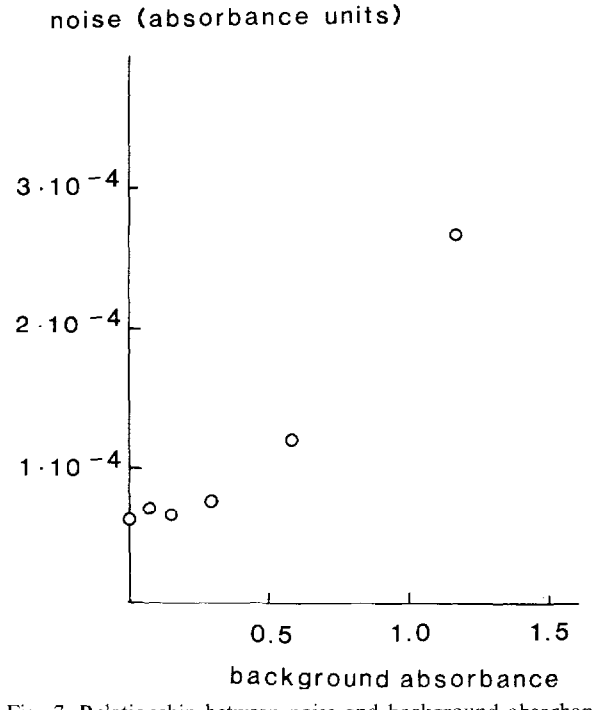

Fig. 7. Relationship between noise and background absorbance. Detector: LDC UV-Monitor III, 254 nm. Absorbing ion: naphthalene-2-sulphonate.

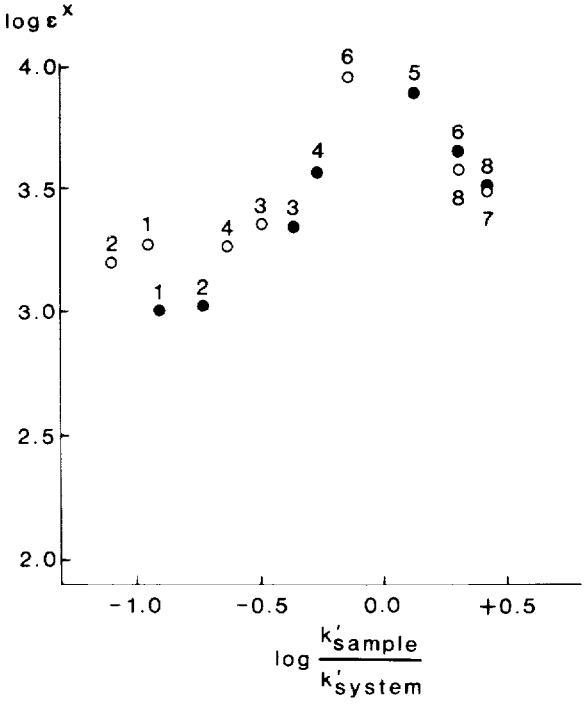

Fig. 8. Two UV-absorbing probes in the mobile phase. Solid phase: μBondapak Phenyl. Mobile phase: ●, $1 \cdot 10^{-4}$ *M* 1-phenethyl-2-picolinium, $5 \cdot 10^{-4}$ *M* 3-hydroxybenzoic acid, $4 \cdot 10^{-4}$ *M* Na^+ in aqueous solution (pH = 6.3); ○, $6 \cdot 10^{-5}$ *M* 1-phenethyl-2-picolinium, $1 \cdot 10^{-4}$ *M* 6-hydroxynaphthalene-2-sulphonate, $4 \cdot 10^{-5}$ *M* Na^+ in aqueous solution (pH = 6.2). Detection: 254 nm. Sample: 1 = pentylamine; 2 = butanesulphonate; 3 = hexylamine; 4 = pentanesulphonate; 5 = heptylamine; 6 = hexanesulphonate; 7 = octylamine; 8 = tetrapropylammonium.

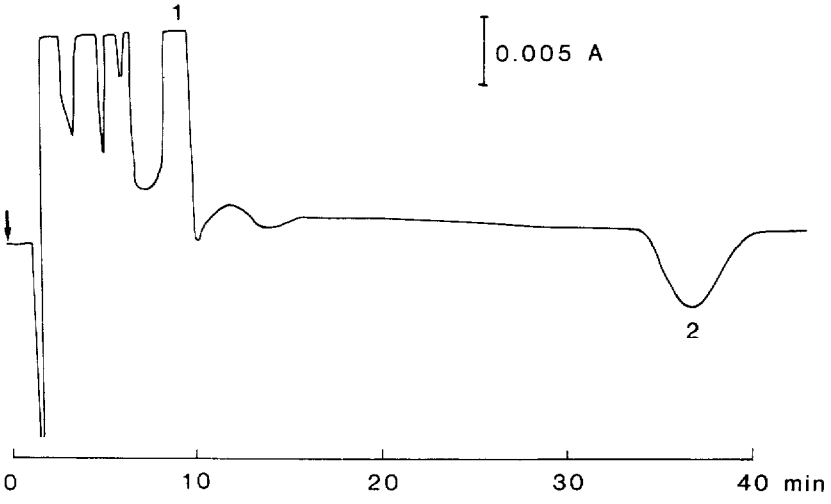

Fig. 9. Sample in complex solvent. Solid phase: μBondapak Phenyl. Mobile phase: $4 \cdot 10^{-4}$ *M* naphthalene-2-sulphonate (sodium salt) in 0.05 *M* phosphoric acid. Solvent: urine, diluted 10-fold with mobile phase. Sample: octanesulphonate. 1, System peak; 2, octanesulphonate.

Quantitation

In the present method, the chromatographic response is due to absorbance deviations from a constant, fairly high background signal, and quantitation of solutes can be made by measuring the peak heights or peak areas of both positive and negative peaks.

The noise level of the background will have a considerable influence on the precision. The noise, measured as the maximum amplitude of the combined short- and long-term absorbance variations, is highly dependent on the background absorbance, as can be seen from Fig. 7. The noise is constant and acceptable up to an absorbance of about 0.5, but increases strongly at higher background. In order to obtain the lowest possible noise, the reservoir, injector, column, and connecting tubes should be thermostated in a water-bath.

The observed molar absorptivity also decreases with increasing absorbance of the mobile phase[9], and this leads to the conclusion that a high background absorbance should be avoided when high sensitivity and precision are needed for an analysis. A detector with the capability of changing the detection wavelength is therefore desirable when using this technique.

The detection sensitivity is fairly low for compounds with considerably lower retentions than that of the system peak (*cf.*, Fig. 3). It can be improved if the mobile phase contains two probes with different charges and hydrophobicities, having absorptivities in the same wavelength range[10]. Two examples are given in Fig. 8. 1-Phenetyl-2-picolinium is used as a hydrophobic cationic probe, and the hydrophilic anionic probes are 6-hydroxynaphthalene-2-sulphonate and 3-hydroxybenzoate, respectively. The conditional molar absorptivity is 1000 or more in a wide retention range. In the system containing 3-hydroxybenzoate, quantitation studies on alkylsulphonates and alkylamines with $k'_{\text{sample}} / k'_{\text{system}}$ between 0.24 and 2.7 showed a linear response for injected amounts from 50 pmol up to 2 nmol, based on peak-height measurements. In peak-area measurements, the linearity increased to about 4 nmol. The samples could be dissolved in either the mobile phase or in water without significantly influencing the result.

The response to a solute seems to be independent of the presence of other retained compounds in the injected sample. Quantitation studies in, *e.g.*, a system with naphthalene-2-sulphonate ($4 \cdot 10^{-4}$ *M* in 0.05 *M* phosphoric acid) as mobile phase and μBondapak Phenyl as solid phase gave linear calibrations for pentylamine ($k'_{\text{sample}} / k'_{\text{system}} = 0.34$) and hexanesulphonate ($k'_{\text{sample}} / k'$system $= 0.51$) up to about 7 nmol, even in the presence of other amines or sulphonates of similar hydrophobicity in the same concentration as the analytes.

Quantitations can even be made in complex matrices such as urine. Fig. 9 shows a chromatogram obtained with octanesulphonate, dissolved in urine, which was diluted 10-fold with the mobile phase. Quantitations gave the same result as for octanesulphonate dissolved in the mobile phase. However, in this matrix, only solutes with a retention higher than that of the system peak ($k' = 10$) can be quantified.

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